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Taxol is a microtubule poison that has been used successfully in refractory breast cancer. Apart from its well characterized anti-mitotic effects, Taxol shares with bacterial lipopolysaccharide (LPS) the capacity to elicit microtubule-independent, intracellular signaling pathways in murine macrophages that activate a serine/threonine and tyrosine kinase cascade that leads to expression of many genes. This IDEA grant proposed to test the ability of Taxol to up-regulate expression of two genes, *nm23* and *c-kit*, whose expression is down-regulated in advanced, metatstatic breast cancer. In addition, modulation of adrenomedulin (AM), a gene expressed by both cancer cells and macrophages, has been examined. To date, we have: (1) optimized conditions for the detection of nm23, c-kit, and AM mRNA by reverse transcriptase polymerase chain reaction (RT-PCR) in murine cells, (2) demonstrated differential expression of nm23 mRNA in two melanoma cell lines (TK1735 2-4 and the nm23 transfectant, TK1735 4-6), and (3) demonstrated that both LPS and Taxol strongly up-regulate expression of AM, and to a lesser extent, c-kit and nm23, in LPS-responsive murine macrophages. We are now ready to move forward with our analysis of breast cancer cell lines as originally proposed.

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Stefanie N. Vogel 7/18/97

PI Signature Date

Stefanie Vogel, Ph.D.

TABLE OF CONTENTS

INTRODUCTION

Taxol is the prototype of a new class of mcrotubule stabilizing agents that has generated great enthusiasm in the oncology community due to its favorable response rate in patients with aggressive, metastatic cancers, including breast cancer (1). In addition to its anti-mitotic effects on cells, which is mediated by its ability to bind to β-tubulin and prevent the microtubule depolymerization required for cell division, Taxol has also been shown in murine macrophages to mimic the action of bacterial lipopolysaccharide (LPS) to modulate gene expression and tyrosine phosphorylation of proteins (2). More recently, Haskill *et al.* (3) have found that Taxol also modulates gene expression in certain primary ovarian tumor cells. For this reason, we hypothesized that Taxol may also exert its anti-tumor effects by modulating the expression of certain genes whose expression is dysregulated in breast cancer. We proposed in our original grant to analyze in both macrophages and breast cancer cells the effects of Taxol and LPS on the expression of two genes, *nm23* and *c-kit*, whose expression has been found to be down-regulated in breast cancer (4,5). Conversely, the gene that encodes adrenomedullin (AM) is expressed in virtually all cancer cells and AM has recently been implicated as an autocrine growth factor in malignant cells (6). For this reason, expression of the AM gene, and its modulation by Taxol and LPS, will also be analyzed in macrophages and breast cancer cells. The main hypothesis to be tested is that Taxol and/or LPS may modulate expression of these genes not only in macrophages, but also in in breast cancer cells, where their expression is dysregulated. Such a modulation could result in restoration of normal homeostatic gene expression.

BODY **(Revised, September 1997)**

In order to initiate the proposed studies, we had to develop the capability of detecting nm23, c-kit, and AM mRNA expression by reverse transcriptase polymerase chain reaction (RT-PCR) technology. Our laboratory has had extensive experience in the use of RT-PCR to assess modulation of gene expression both *in vitro* and *in vivo* of a broad panel of inflammatory and anti-inflammatory genes and the specific methodology employed in this approach has been detailed elsewhere (7,8). Sense and antisense oligonucleotide primer pairs and probes were selected, based on the published nucleotide sequences of nm23, c-kit, and AM and the conditions for detection of these genes were optimized during this initial funding period. Table 1 ("Optimized conditions for analysis of genes of interest by RT-PCR") summarizes not only the sequences required to identify each mRNA species specifically, but also the expected and confirmed PCR product sizes, conditions of annealing temperature, and cycle number for RNA extracted from each cell type examined. Sense and anti-sense primer sequences were selected to insure that specific RNA could be distinguished from contaminating genomic DNA (7). As you will note from Table 1, two sets of sequences have been used to detect nm23 mRNA and are designated "NM23" and "NM23(L)." At the time this proposal was originally submitted, only one nm23 cDNA sequence was present in the Genbank. Since

starting this project, we found that a second, longer (L) sequence had been submitted to the Genbank. Thus, "NM23" refers to nucleotide sequences that were based on the original cDNA sequence, whereas "NM23(L)" refers to those that detect the "long" form selectively. Thus, we had anticipated the possibility that multiple isoforms of of nm23 might exist. However, to date, we have observed no differences in the use of these two pairs of primer sets, as would be expected if only a single gene product were expressed at the level of mRNA. An example of semi-quantitative RT-PCR analysis is provided in Figure 1A.

As a positive control for nm23 mRNA expression, we obtained from Dr. Patricia Steeg (NCI, NIH) two murine melanoma cell lines, TK1735 2-4 and 4-6; the latter has been stably transfected with and overexpresses the *nm23* gene. In addition, we analyzed mRNA prepared from the murine macrophage cell line, RAW 264.7, as well as mRNA derived from primary murine macrophage (C3H/OuJ) cultures. The expression of nm23 mRNA in TK1735 4-6, the cell line stably transfected with the *nm23* gene, was significantly greater than in the control cell line, TK1735 2-4. This can be seen in Figure 1B, by comparing the relative intensity of the mRNA signals in TK1735 2-4 (left panel; medium) to that of TK1735 4-6 (right panel; medium), under conditions where expression of the housekeeping gene, HPRT, is extremely comparable. Optimization of conditions for detection of the "housekeeping" gene, HPRT, as an internal control for variation in the levels of cDNA produced from sample to sample, has been previously reported by this laboratory (7). In contrast, neither c-kit nor AM mRNA species were expressed in the melanoma cell lines with up to 40 cycles of PCR amplification (see Table 1).

In addition to nm23, both c-kit and AM mRNA were constitutively expressed in the primary macrophage mRNA preparations. In contrast, the macrophage cell line, RAW 264.7, failed to express c-kit mRNA, although constitutive expression of both nm23 and AM mRNA species was detected (Table 1). Thus, all of the molecular methodology required to complete the remaining proposed aims of this IDEA project is now established in our laboratory.

We next sought to determine if the malignant cell lines provided to us by Dr. Steeg or if the RAW 264.7 or C3H/OuJ macrophages responded to Taxol or LPS to modulate expression of nm23, c-kit, or AM mRNA levels. Cells were stimulated with medium only, LPS, or Taxol over an extended timecourse (24 hours) and mRNA was extracted at closely spaced intervals. The concentrations of LPS (100 ng/ml) and Taxol (35 μM) chosen for these studies were based on previous observations from our laboratory and reflect the optimal stimulatory concentrations for macrophages (2). Both Figure 1B and Figure 2 illustrate that neither TK1735 2-4 (control) nor TK1735 4-6 (nm23-transfected) cell lines responded to LPS or Taxol to increase expression of nm23 mRNA significantly over levels expressed constitutively (designated as "1" on the y-axis of Figure 2). However, the failure of these melanoma cell lines to respond to LPS or Taxol to modulate nm23 mRNA expression is not necessarily a predictor of what we will see when breast cancer cells are similarly treated. As stated above, the melanoma cell lines that we obtained from Dr. Steeg were used initially as a positive control to insure that we were truly

detecting nm23 mRNA species. Expression of all three genes will be evaluated in the near future in normal and breast cancer cell lines cultured with medium only, LPS, or Taxol. We have recently obtained some of these cell lines and have been optimizing culture conditions for their growth and maintenance during the past several months.

As was observed in the malignant cell lines, nm23 mRNA levels were also not modulated in the RAW 264.7 cell line stimulated with Taxol or LPS over a 24 hour culture period (Figure 3). In contrast, LPS and Taxol induced in the primary macrophages a 2-5-fold increase in nm23 mRNA over background levels in two of three separate experiments, with elevated levels being sustained for up to 24 hr after stimulation of cultures (data not shown). The failure to detect nm23 RNA in the macrophage cell line, in the face of a variably detectable increase in nm23 RNA from primary macrophage cell cultures, may be related either to differences in the differentiation state of the primary vs. the cell line macrophages or possibly to the fact that the RAW 264.7 cell line is transformed and actively dividing, in contrast to the primary cell cultures. Future experiments, apart from the scope of this study, will be required to distinguish between these possibilities.

The effects of LPS and Taxol on the expression of c-kit mRNA in C3H/OuJ macrophages were difficult to evaluate since basal expression of this gene seemed to be upregulated transiently when the cells were cultured with medium only. Figure 4 illustrates that although there appears to be a slight up-regulation of c-kit mRNA expression in response to LPS and Taxol, a trend that paralleled expression of this mRNA species in medium only, induced levels were not statistically significant (Figure 4).

This weak pattern of gene expression is in striking contrast to the results we have obtained in macrophages in which AM mRNA was measured following treatment with Taxol or LPS. Briefly, Figure 5 illustrates very clearly the inducibility of AM mRNA by LPS and Taxol in primary macrophages: induction is brisk, peaking within 2 hours, and reaching levels that are 10-15-fold above baseline. Both Taxol and LPS were potent inducers of AM gene expression. By comparison, IFN-γ, another potent macrophage stimulant, also increased AM mRNA expression, albeit more sluggishly (peaking at about 4 hours) and to a lesser extent (approximately 7-fold above background; Figure 6). As an additional control, primary macrophages derived from the LPS-hyporesponsive C3H/HeJ background, that possess a defect in the capacity to respond to purified, protein-free LPS or Taxol (2), were found to respond to a variety of non-LPS stimuli to express AM mRNA (Figure 7). These data were recently presented in poster form at an adrenomedullin conference that was held at NIH earlier this month. Because a significant increase in AM mRNA was observed, analysis of AM protein levels in culture supernatants are presently being measured by radioimmunoassay by Dr. Frank Cuttitta and his colleagues at the NCI, NIH. Preliminary data suggest that in addition to the observed increase in mRNA induced in primary macrophages by LPS or Taxol, a significant release of AM protein is observed. We have begun preparation of a manuscript that will describe our

findings on the regulation of AM mRNA and protein by LPS, Taxol, and IFN-γ in primary murine macrophage cultures.

CONCLUSIONS

During the first year of funding on this project, we have developed all of the molecular methodology required to evaluate the effects of Taxol and LPS on the specific expression of nm23, c-kit, and AM mRNA in murine macrophages and in breast cancer cell lines. In addition, differential expression of the three genes in control melanoma cells and in the macrophages was observed. Taxol and LPS up-regulated expression of AM, and to a lesser extent, c-kit and nm23, in macrophage cultures. We are nearing completion of the studies analyzing mRNA from macrophages for their ability to be stimulated by LPS or Taxol to express genes that have been reported in the literature to be either repressed (e.g., nm23 or c-kit) or augmented (e.g., AM) in breast cancer cells. Now that we are confident that we are able to detect all of the genes of interest, and that Taxol and LPS modulate their expression differentially in the different control cell types, we are poised to move forward to an analysis of the effects of Taxol and LPS on breast cancer cell lines, as described in the original proposal. We have recently obtained breast cancer cell lines and will soon analyze the responsiveness of these cells to LPS, Taxol, and IFN-γ to modulate expression of all three genes. We are confident that we will complete all of the aims of this proposal within the upcoming funding period.

To date, we have completed, in part or in entirety, Tasks 1a, b and Tasks 2a, b, and c. The development of additional nm23-specific oligonucleotide probes (based on the recently published sequence of a longer form of nm23) and the unanticipated need to verify the efficacy of our nm23 primer sets with known positive controls (e.g., TK1735 2-4 and 2-6 melanoma cells), coupled with the recent discovery of overexpression of AM in malignant cells and cell lines (6), led to some additional experiments that were not initially proposed. However, they have already yielded important findings, and will not preclude the completion of the remaining Tasks 1c-g, as originally proposed. It should be noted that our funding was delayed, in that it was made available to us approximately a month later than anticipated. Only when the funds were made available to us could I advertise for the personnel required for this grant and it took several months to identify and bring on board the best person for this study. Nonetheless, with all of the molecular reagents in hand, and nearing completion of the studies with macrophages, I am optimistic that we can complete our analysis of nm23, c-kit, and AM expression in breast cancer cells during the second funding period of this grant.

REFERENCES

1. Rowinsky EK, N Onetto, RM Canetta, and SG Arbuck. 1992. Taxol: the first of the taxanes, an impoarant new class of antitumor agents. *Semin Oncol* 19: 646-662.

2. Manthey CL, ME Brandes, P-Y Perera, and SN Vogel. 1992. Taxol increases steady-state levels of LPS-inducible genes and protein-tyrosine phosphorylation in murine macrophages. *J Immunol* 149: 2459-2465.

3. Lee L-F, C-C Schuerer-Maly, AK Lofquist, C van Haaften-Day, JP-Y Ting, CM White, BK Martin, and JS Haskill. 1996. Taxol-dependent transcriptional activation of IL-8 expression in human ovarian cancer. *Cancer Res* 56: 1303-1308.

4. Steeg PS, A De La Rosa, U Flatow, NJ MacDonald, M Benedict, and A Leone. 1993. Nm23 and breast cancer metastasis. *Breast Cancer Res and Treatment*25: 175-187.

5. Natali G, MR Nicrtra, I Sures, M Mottolese, C Botti, and A Ullrich. 1992. Expression of *C-kit* receptor in normal and transformed human nonlymphoid tissues. *Cancer Res* 52: 6139-6143.

6. Miller MJ, A Martinez, EJ Unsworth, CJ Thiele, TW Moody, T Elsasser, and F Cuttitta. 1996. Adrenomedullin expression in human tumor cell lines. Its potential role as an autocrine growth factor. *J Biol Chem* 271: 23345-23351.

7. Salkowski CA, R Neta, TA Wynn, G Strassmann, N van Rooijen, and SN Vogel. 1995. Effect of liposome-mediated macrophage depletion on LPS-induced cytokine gene expression and radioprotection. *J Immunol* 155: 3168-3179.

8. Wynn T, I Eltoum, AW Cheever, FA Lewis, WC Gause and A Sher. 1993. Analysis of cytokine mRNA expression during primary granuloma formation induced by eggs fo *Schistomsoma mansoni. J Immunol* 151: 1430-1440.

APPENDICES:

Table 1
Figure legends
Figures 1A, 1B, 2, 3, 4, 5, 6, 7

Table 1. Optimized conditions for analysis of genes of interest by RT-PCR

Gene	Oligonucleotide sequence	Product size	Annealing temperature	Cycle number TK	RAW	C3H/OuJ
NM23	S: 5'-TGAATGTGGTGAAGACAG	257	50°C	23	26	28
	AS: 5'-AGAACAAGAGTAAGCAGGTAG					
	P: 5'-GAGAGACCAACCCCGCAGAC					
NM23(L)	S: 5'- TGGATAACCTGAGTGGAC	172	50°C	26	30	28
	AS: 5'-ACAACCTCACACATCCTC					
	P: 5'-GAGCCACCACCCTGAGACAC					
c-Kit	S: 5'-CAGAAACCCATGTATGAAGTA	752	52°C	—	—	32
	AS: 5'-TGAATACAATTCTTGGAGGC					
	P: 5'-CGGAACTGAAGGTCCTGAG					
AM	S: 5'-AAGAAGTGGAATAAGTGGGCG	267	55°C	—	35	28
	AS: 5'-ACCAGATCTACCAGCTA					
	P: 5'-CCCCCTACAAGCCAGCAATCAG					

S=sense, AS=antisense,P=probe,--- = the specific gene is not expressed in these cells.

FIGURE LEGENDS

Figure 1. (A) Example of semi-quantitative RT-PCR analysis as carried out in our laboratory. This method has been detailed in References 7 and 8. (B) RT-PCR of nm23 mRNA from TK1735 2-4 and TK1735 4-6 melanoma cells stimulated for 2 hr with medium only, Taxol, or LPS. Simultaneous amplification of the housekeeping gene, HPRT, serves as an internal control for cDNA expression (7,8).

Figure 2. Induction of NM23(L) mRNA expression in melanoma TK1735 2-4 (control, non-transfected with NM23) and TK 1735 4-6 (NM23-transfected) cells after LPS or Taxol stimulation. Cells were treated with medium only, 100 ng/ml LPS or 35 μM of Taxol for the indicated periods of time. RNA was isolated, and NM23(L) and HPRT mRNA were detected by RT-PCR at 28 and 24 cycles, respectively. Data are from a single representative experiment (n = 3).

Figure 3. Induction of NM23 mRNA expression in RAW 264.7 macrophages after LPS or Taxol stimulation. Cells were treated with medium only, 100 ng/ml LPS, or 35 μM Taxol for the indicated periods of time. RNA was isolated and NM23 and HPRT mRNA were detected by RT-PCR at 28 and 24 cycles, respectively. Analysis of HPRT indicated that equivalent amounts of mRNA were reverse transcribed into cDNA. Data are derived from 4 separate experiments.

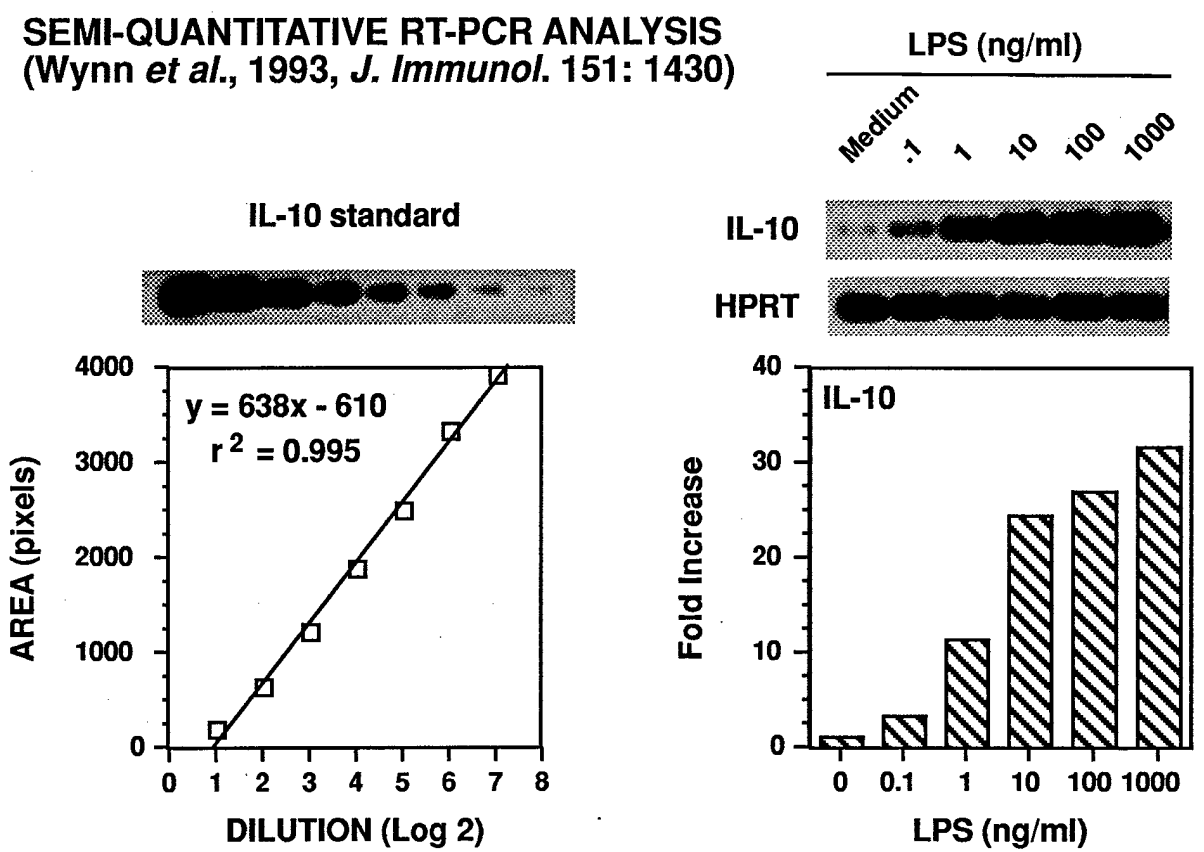
Figure 4. Induction of c-kit mRNA expression in C3H/OuJ macrophages after LPS or Taxol stimulation. Cells were treated with medium only, 100 ng/ml LPS, or 35 μM Taxol for the indicated periods of time. RNA was isolated and c-kit and HPRT mRNA were detected by RT-PCR at 32 and 24 cycles, respectively. Data are derived from 4 separate experiments.

Figure 5. Induction of AM mRNA expression in macrophages after LPS or Taxol stimulation. C3H/OuJ macrophages were treated with medium only, 100 ng/ml LPS, or 35 μM Taxol for the indicated periods of time. RNA was isolated and AM and HPRT mRNA were detected by RT-PCR at 28 and 24 cycles, respectively. Analysis of HPRT indicated that equivalent amounts of mRNA were reverse transcribed into cDNA. Data are from a single representative experiment (n = 7).

Figure 6. Induction of AM mRNA expression in C3H/OuJ macrophages by IFN-γ. C3H/OuJ macrophages were cultured with 10 U/ml IFN-γ for 0, 2, 4, 6, and 24 h. RNA was isolated and AM and HPRT mRNA were detected by RT-PCR at 28 and 24 cycles, respectively . Data are from a single representative experiment (n = 3).

Figure 7. C3H/HeJ macrophages were treated for 4 hours with medium only, STAg (5 μg/ml), MeXAA (10 μg/ml), But-LPS (5 μg/ml; shown previously to stimulate C3H/HeJ macrophages due to the presence of contaminating LPS-associated proteins), or highly purified, protein-free PW-LPS (100 ng/ml; shown previously <u>not</u> to stimulate C3H/HeJ macrophages). The data represent the mean ± SEM from 3 separate experiments.

SEMI-QUANTITATIVE RT-PCR ANALYSIS
(Wynn *et al.*, 1993, *J. Immunol.* 151: 1430)



Calculating gene expression using RT-PCR

1. A standard positive sample is diluted two-fold serially and assayed by RT-PCR (see text). The autoradiograph can be scanned densitometrically. The data are used to create a best line of fit relating the dilution of the sample to pixel density (left panel).

2. The experimental samples are also assayed by RT-PCR simultaneously (right panel) and the pixel density of their signals is measured by scanning densitometry and then related to the standard curve.

3. Each of the samples of interest is also assayed for the "housekeeping gene," HPRT, similarly to that described for IL-10. An IL-10/HPRT ratio can then be calculated. HPRT expression does not change with LPS treatment, so it becomes an internal control for lane to lane loading differences.

4. Once the ratios of IL-10/HPRT are calculated for the different treatment groups, they are all divided by the value obtained in the untreated control. This gives a "fold increase" (e.g., the increase over the background control).

Fig. 1A

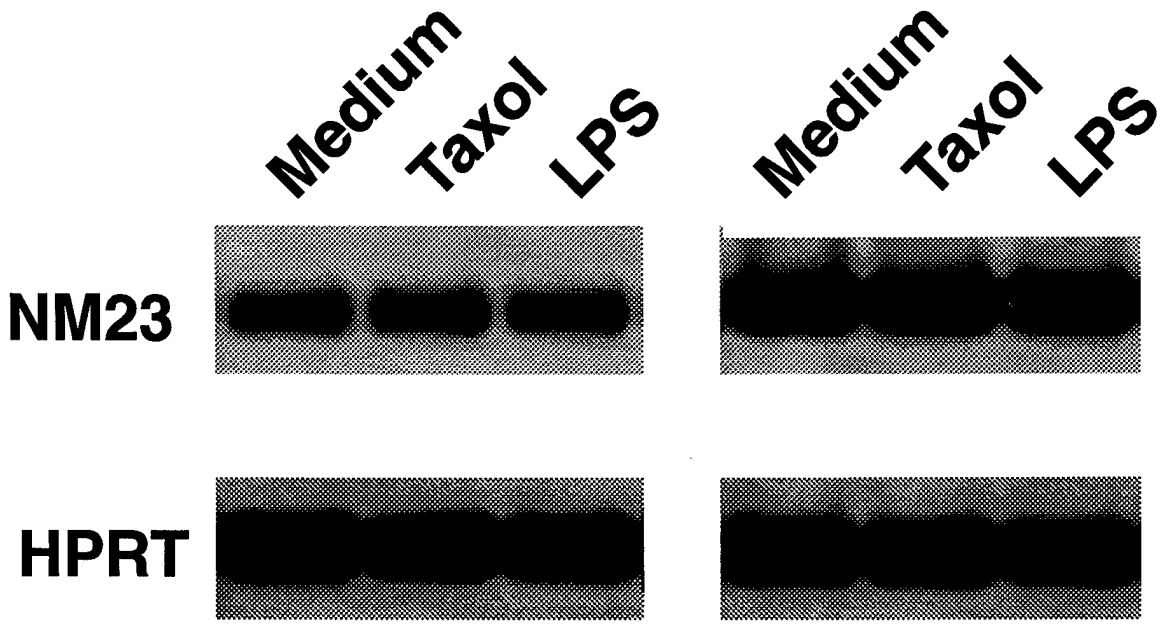

TK 1735 2-4
TK 1735 4-6
Medium
Taxol
LPS
Medium
Taxol
LPS
NM23
HPRT

Figure 1B

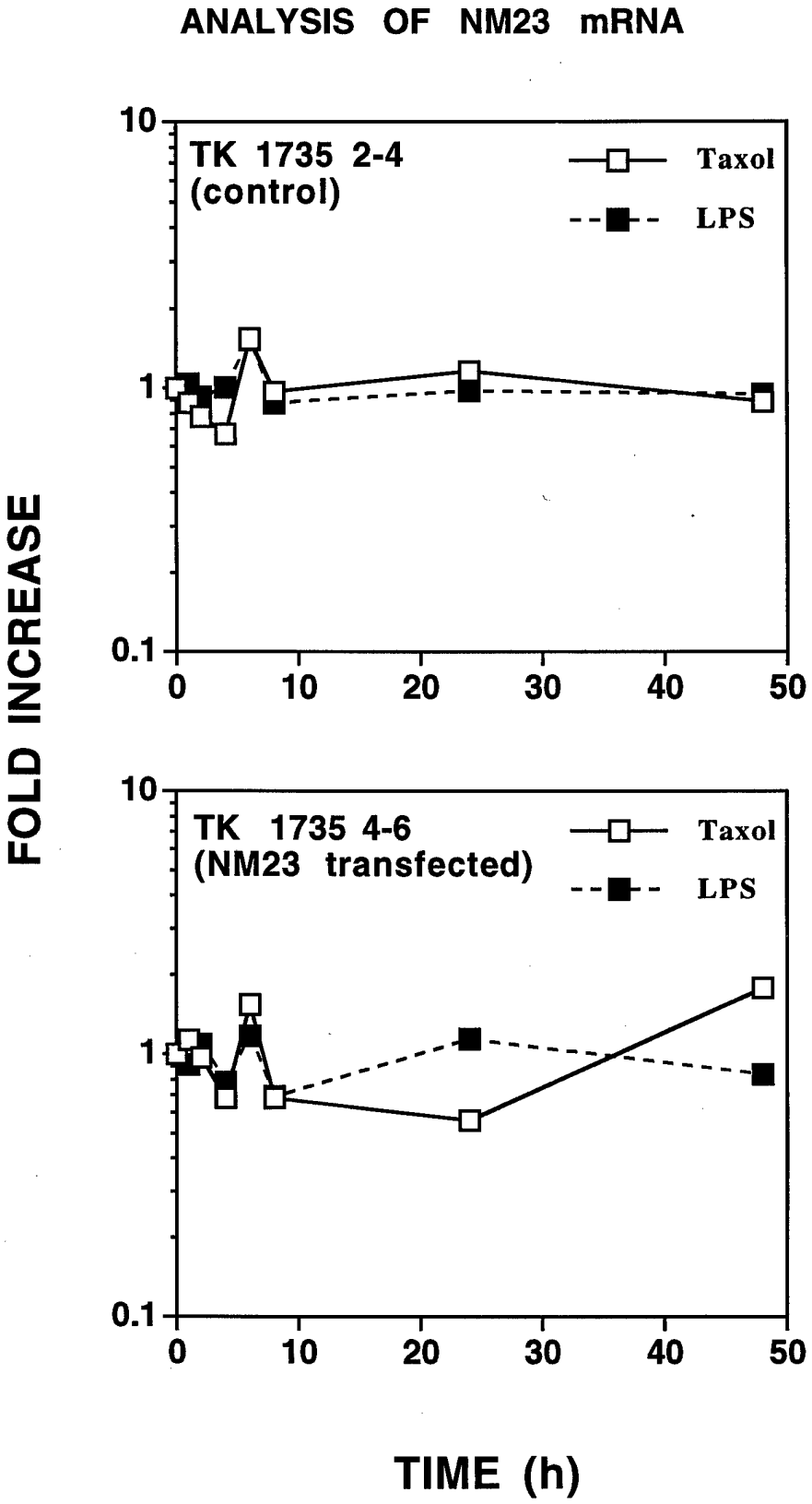

ANALYSIS OF NM23 mRNA
TK 1735 2-4
(control)
Taxol
LPS
TK 1735 4-6
(NM23 transfected)
Taxol
LPS
FOLD INCREASE
10
1
0.1
0
10
20
30
40
50
TIME (h)

Figure 2

ANALYSIS OF NM23 mRNA IN THE RAW 264.7 MACROPHAGE CELL LINE

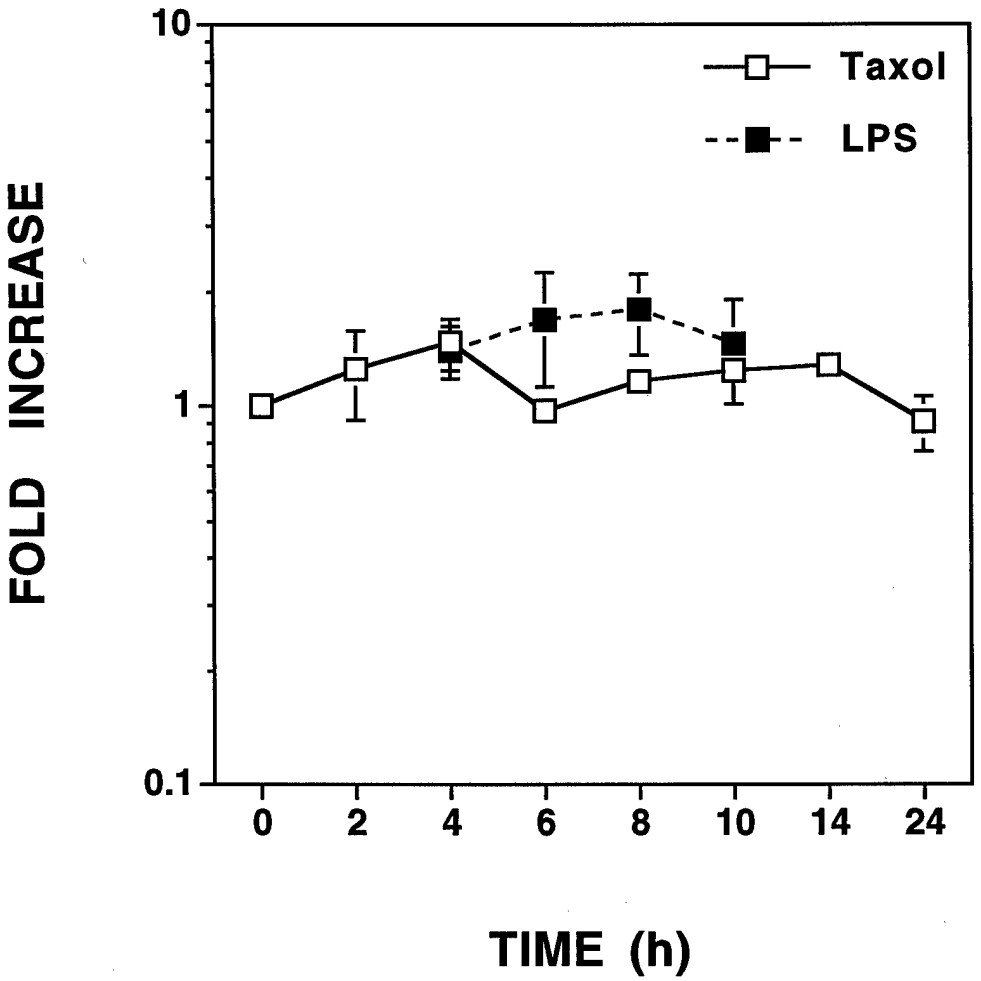


Figure 3

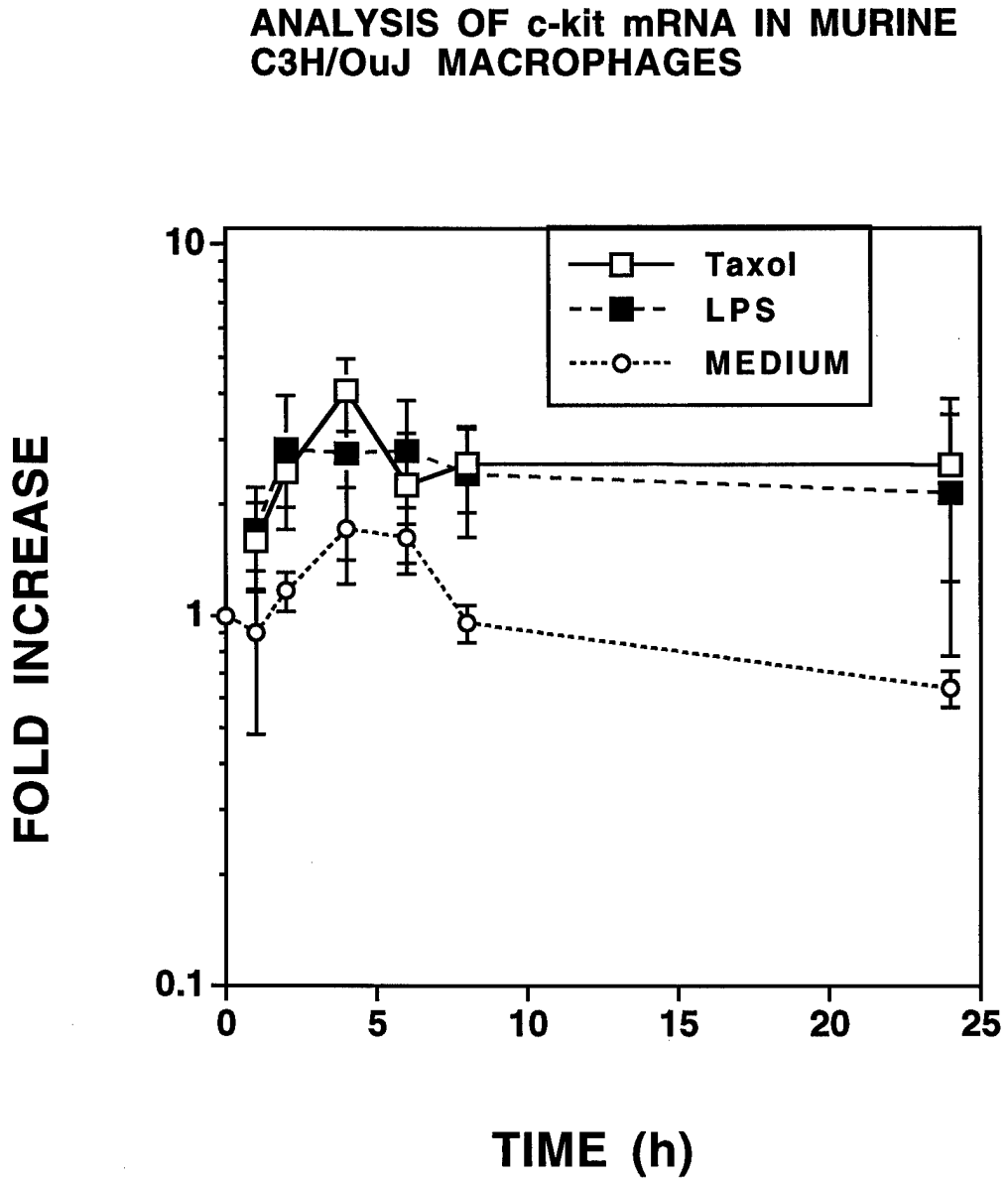

ANALYSIS OF c-kit mRNA IN MURINE C3H/OuJ MACROPHAGES
FOLD INCREASE
10
1
0.1
0
5
10
15
20
25
TIME (h)
Taxol
LPS
MEDIUM

Figure 4

KINETICS OF AM mRNA INDUCED BY LPS OR TAXOL IN MURINE MACROPHAGES

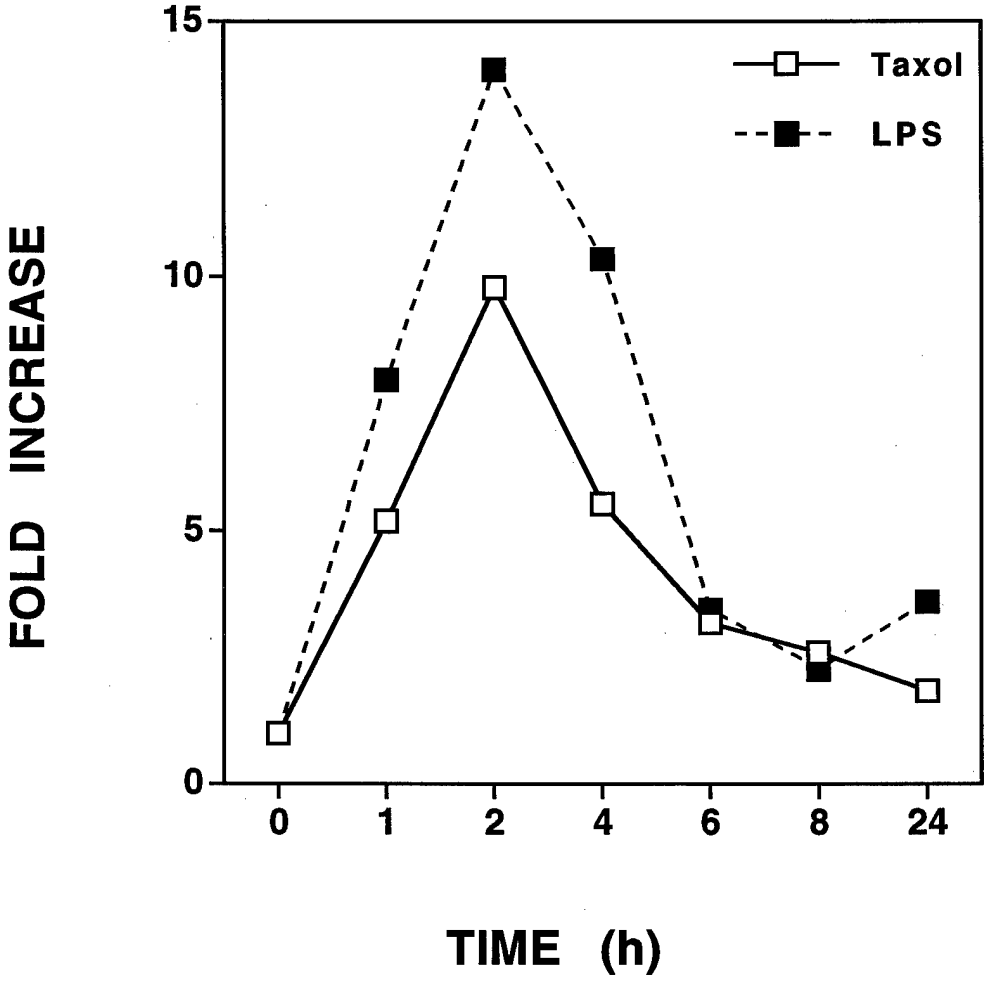


Figure 5

KINETICS OF AM mRNA INDUCED BY IFN-γ IN MURINE MACROPHAGES

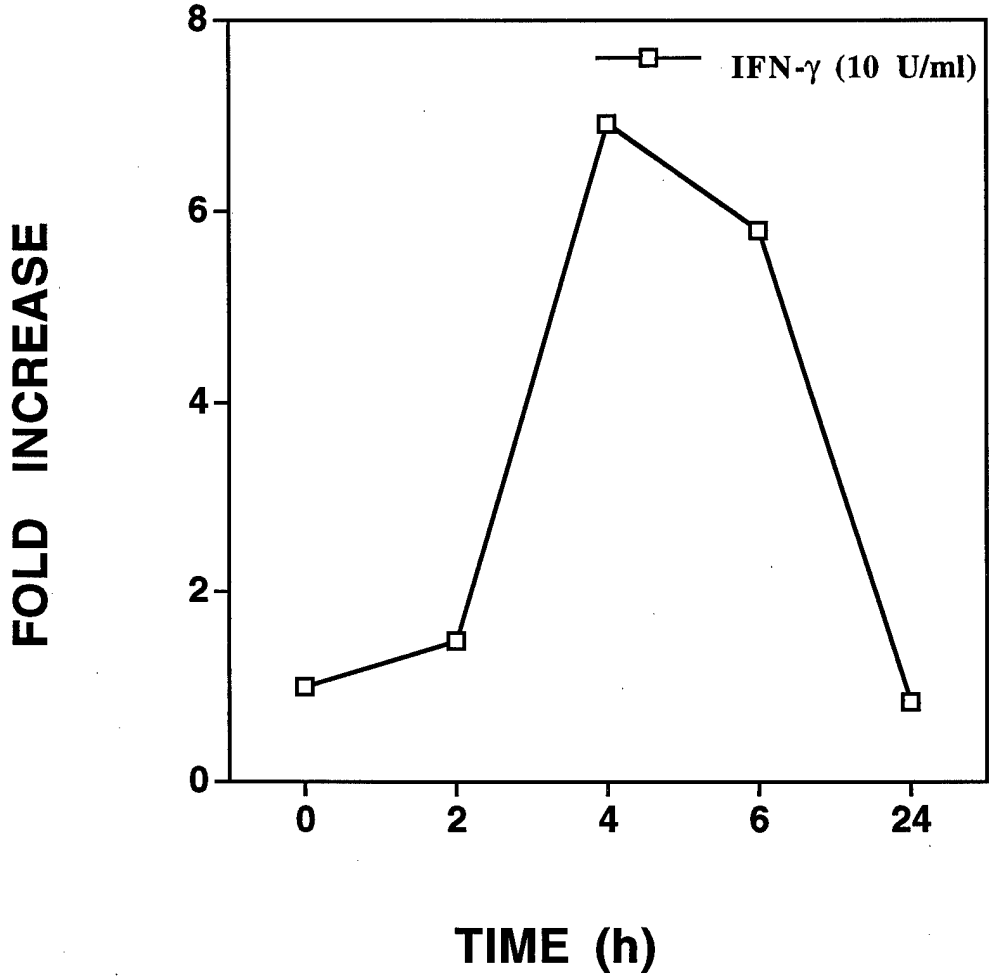


Figure 6

INDUCTION OF AM mRNA BY STAg, MeXAA, BUT-LPS, AND PW-LPS IN C3H/HeJ MACROPHAGES

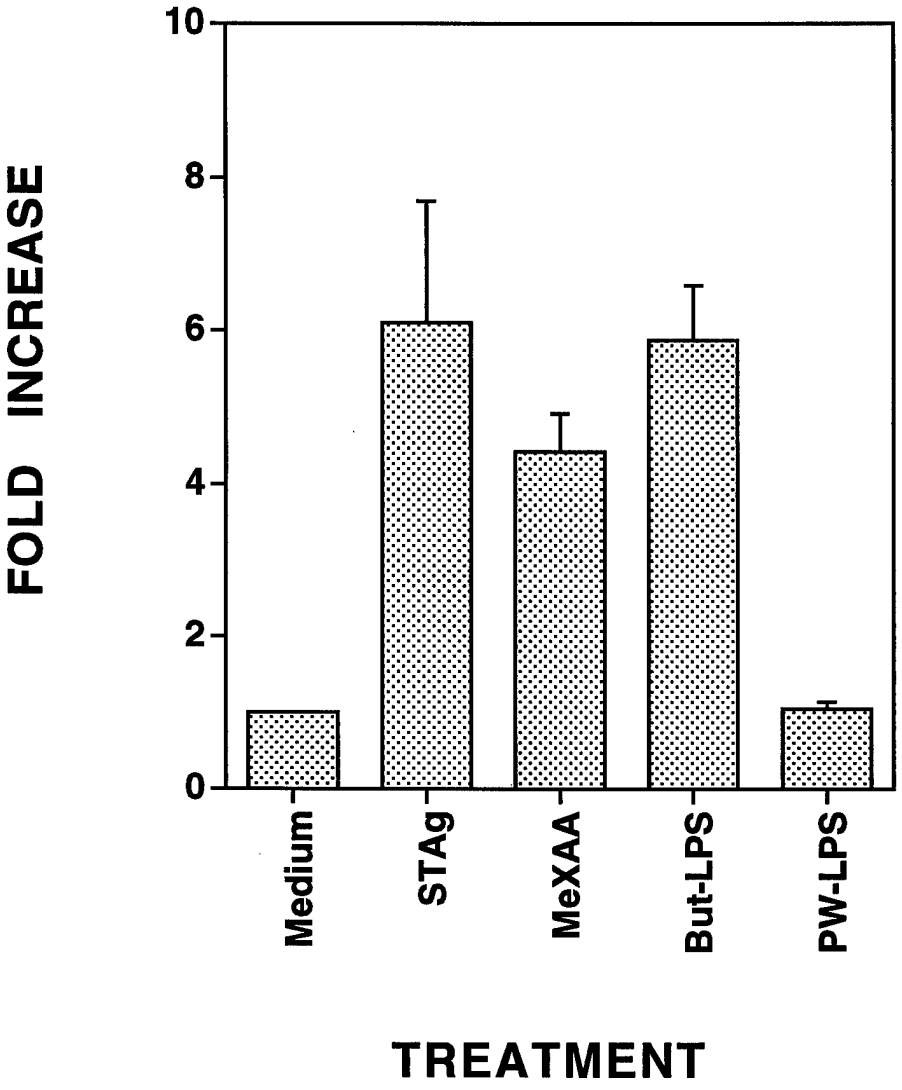


Figure 7